

**Observations of Over 11 Years in Recipients of Combined HLA mismatched  
Kidney and Bone Marrow Transplantation Without Maintenance  
Immunosuppression**

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**Contribution:**

Tatsuo Kawai, A. Benedict Cosimi, David H. Sachs, Megan Sykes, Thomas Spitzer and Robert B. Colvin performed study design, clinical management, data analyses and manuscript writing. Peter Sayer performed study design. Nina Tolloff-Rubin, Waichi Wong, Winfred W. Williams, Kerry Crisalli and Rex-Neal Smith participated in clinical management. Susan L. Saidman performed DSA assays. Fred Pfeffer performed lymphocyte subset assays, Baoshan Gao and Emmanuel Zorn performed serum BAFF measurement. Ben Sprangers, Heather Morris, Brittany Shonts and Samuel LoCascio, with supervision from Megan Sykes, performed in vitro assays and data analyses.

**Abstract:**

We report here the long-term results of HLA mismatched kidney transplantation without maintenance immunosuppression (IS) following combined kidney and bone marrow transplantation (CKBMT). The first three subjects were treated with nonmyeloablative conditioning and an 8-14 month course of calcineurin inhibitor (NKD03). The next two subjects received, in addition, two doses of pre-transplant rituximab (mod NKD03). The last five recipients received two pre and two post-transplant rituximab doses (ITN036). All 10 subjects developed transient chimerism and in seven of these, IS was successfully discontinued. Four subjects continue to be IS free for periods of 4.3-11.2 years, while three required IS after 6-8 years due to recurrence of original disease or chronic allograft glomerulopathy. Donor-specific antibody (DSA) was detectable in some recipients treated with the NKD03 and mod NKD03 regimens. In contrast, no DSA was detected in ITN036 subjects, who showed more consistent and durable B cell depletion, despite high BAFF levels. In conclusion, long-term stable kidney allograft survival without maintenance IS can be achieved following transient mixed chimerism induction. Adequate B cell depletion during the initial 6 months appears to be an important factor in preventing development of de novo DSA.

**Introduction:**

Short-term results following organ transplantation have been significantly improved by the use of increasingly efficacious immunosuppressive agents. However, their chronic administration results in significant morbidity, especially from an increased incidence of cardiovascular disease(1), infection(2), malignancies(3), de novo diabetes(4) and other metabolic derangements. Unfortunately, the potent immunomodulatory effects of current therapeutic protocols do not prevent the development of chronic rejection, despite their administration being pushed to toxic levels. Therefore, induction of tolerance, defined as the absence of destructive immune responses to a transplanted tissue without ongoing immunosuppressive therapy, remains the ultimate goal of organ transplantation.

Since the seminal work reported by Billingham, Brent and Medawar on neonatal tolerance in 1956, numerous tolerance induction strategies have been defined in rodents. However, only a very limited number of these strategies have been successfully translated to large animals and even fewer to primates. Among the few protocols that have been applied successfully in humans, induction of donor chimerism, either transient or permanent, currently appears to be the most promising strategy to achieve renal allograft tolerance. Initial results of clinical trials for tolerance induction in three centers have so far been reported. Using TLI and DBMT, the Stanford group reported successful induction of stable chimerism and renal allograft tolerance in the majority of HLA-identical kidney transplant recipients(5-7). More recently, Leventhal et al. at Northwestern have

reported the use of an intensive conditioning regimen and donor hematopoietic stem cells for induction of full donor chimerism and tolerance in HLA-mismatched kidney transplant recipients(8). Although the follow-up of these patients is still relatively brief, persistent donor chimerism without GVHD has been reported, allowing weaning from all maintenance immunosuppression by 1 year in approximately half of the patients.

At Massachusetts General Hospital, based on decades-long basic studies in animal models(9-12), we have applied combined kidney and donor bone marrow transplantation (CKBMT) for induction of allograft tolerance in both HLA matched(13-15) and HLA-mismatched(16,17) kidney transplant recipients. In this report, we summarize our experience in all 10 subjects, present assays of their anti-T cell responses, B cell depletion and BAFF levels and evaluate the potential relationship between the conditioning regimens and long-term humoral responses. Our observations emphasize the importance of adequate B cell depletion during the initial 6 months to inhibit de novo DSA.

## **METHODS**

### **Study subjects:**

A total of 10 subjects, age 22-46, 6 males and 4 females, were enrolled into these studies. Their original kidney diseases include Alport's syndrome (n=4), polycystic kidney disease (n=2), membrano-proliferative glomerulonephritis (MPGN) type 1(n=2), reflux uropathy (n=1) and focal glomerulosclerosis (n=1) (Table 1). The first three subjects (#1 - #3) received the NKD03 conditioning

regimen; the next two subjects (#4 and #5) received the modified NKD03 regimen. The last five subjects(#6 - #10) received the ITN036 protocol detailed in Fig. 1.

**Conditioning Regimen:** The initial conditioning regimen (Fig. 1, NKD03) consisted of cyclophosphamide (60 mg/kg/d) administered i.v. on days –5 and –4 with respect to transplantation; humanized anti-CD2 mAb (MEDI 507, MedImmune Inc., Gaithersburg, MD) at a test dose of 0.1 mg/kg on day -2 followed by 0.6 mg/kg/dose on days -1, 0 and +1; cyclosporine A (CyA, Novartis Pharmaceuticals Inc., East Hanover, NJ) (5 mg/kg) i.v. on day –1; and thymic irradiation (700 cGy) on day –1. Hemodialysis was performed before and 14 hours after each dose of cyclophosphamide. On Day 0, kidney transplantation was followed by i.v. infusion of unprocessed donor bone marrow (DBM). Oral CyA (Neoral, Novartis Pharmaceuticals, Inc.) was administered postoperatively at 8 to 12 mg/kg/day with target trough blood levels of 250-350 ng/ml, then tapered and discontinued over several months.

The protocol was modified after treatment of the third subject (see Results), with the addition of Rituximab, 375 mg/m<sup>2</sup>/dose on days -7 and -2; and prednisone, 2 mg/kg/d starting on the day of transplantation and tapering to withdrawal over the next 10 post-transplant days (Fig. 1, modified NKD03). Since subjects treated with this modified NKD03 regimen still developed donor-specific antibodies (DSA) after discontinuation of immunosuppression, the regimen was further modified (Fig.1) to add two more doses of Rituximab (375 mg/m<sup>2</sup>/dose) on days 5 and 12, plus a more prolonged course of prednisone until day 20, and

tacrolimus in place of CyA (ITN036). Tacrolimus was slowly tapered over several months and completely discontinued at 8 months after confirming no rejection by a 6 month protocol biopsy. All treatment regimens were approved by the Massachusetts General Hospital Institutional Review Board (IRB) and developed in collaboration with the Immune Tolerance Network (ITN).

**Biopsies.** Kidney allograft biopsies were taken per protocol at day 0 and at months 6, 12, 24 and 36. Some subjects had later protocol biopsies after 5-8 years of follow-up. Indication biopsies were taken for any episode of graft dysfunction. All biopsies were processed for routine light microscopy, immunofluorescence (including C4d stains) and electron microscopy by techniques previously reported (16, 18).

**In Vitro Immunologic Assays:** Standard MLR and CML assays were performed using the methods detailed previously.(14) LDA to quantify cytotoxic T-lymphocyte precursor frequencies and IL-2-producing Th frequencies were performed as described(19).

**Detection of donor-specific antibodies:** Serially collected pre- and post-transplant sera samples were tested for the presence of HLA antibodies using ELISA kits (LAT Class I & II, One Lambda, Canoga Park, CA).

**Serum BAFF measurement:** Concentration of BAFF in the serum was measured using a Quantikine ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Serum samples were diluted 1:8 in PBS.

## RESULTS:

**Induction of transient chimerism:** As in the previously reported patients, all additional subjects developed transient multilineage mixed chimerism, which became undetectable by 2-3 weeks post-CKBMT (16, 20).

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**Acute kidney injury (AKI) 10-14 days after DBMT:** We previously described the cytokine syndrome-like manifestations observed after CKBMT as “engraftment syndrome” (16, 18). In fact, the symptoms have been temporally associated with the loss of peripheral chimerism as well as of the return of host-derived hematopoietic elements. The most troublesome manifestation of this syndrome, and the only manifestation not alleviated by addition of steroids to the post-transplant treatment regimen, was acute kidney injury (AKI), which was observed after day 10 in all patients, although minimally in Subject #1. The biopsies taken during AKI showed endothelial injury with CD8<sup>+</sup> T cell infiltration, as reported previously in detail(18). The peak serum creatinine (Cr.) level ranged from 3-5 to 15-4 mg/dl during days 10-20. Among the ten subjects who developed AKI, four recovered without additional treatment. Renal allograft function was normalized in two subjects in conjunction with Thymoglobulin and plasma exchange, in one with Thymoglobulin alone and in one with additional steroid therapy. Two kidney allografts failed to recover, one in association with humoral rejection due to preformed anti-donor antibody (Subject #3) and one that developed thrombotic micro-angiopathy associated with high tacrolimus levels (Subject #8).

**Long-term clinical course of subjects in NKD03 (Subjects #1-5):**

Initial results, after follow-up periods of up to 2-5 years, in the first five patients, were previously reported(16). As in that report, except for Subject #3, who was retrospectively found to have DSA prior to transplant and suffered early graft failure due to acute antibody-mediated rejection, all renal allografts are functional

with the longest survival now exceeding 11 years (Subject #1). Four protocol biopsies performed in Subject #1 up to 7.5 years after CKBMT showed no evidence of rejection, including the absence of C4d deposition (Figs. 3A-C). Subject #2 was successfully tapered off of IS by 14 months, after treatment for suspected humoral rejection (transient C4d deposition without DSA) at around day 45. He remained stable until year 7 following immunosuppression withdrawal (Fig. 2). At that point, urinary protein first became detectable. Allograft biopsy revealed no rejection but recurrence of his original disease, MPGN type I (C3 glomerulopathy) (Figs. 3D-F). Mycophenolate mofetil (MMF) monotherapy for recurrent MPGN was initiated after the 8<sup>th</sup> year. Although he has been inconsistently compliant, he is currently stable (serum creatinine 2.1 mg/dl) (Fig. 2) with minimal urinary protein (200-300 mg/L). Subject #3 had shown high PRA (50%) but did not show DSA by ELISA and crossmatch was negative before transplantation. However, he lost his renal allograft with severe acute humoral rejection on day 10 (Fig. 2). Retrospective analysis with LUMINEX revealed preformed DSA against HLA class I (FIG. 4A) and his humoral rejection was retrospectively concluded to be due to preformed DSA. This subject subsequently underwent successful second transplantation with conventional IS. Subject #4 received the modified NKD03 regimen (Fig. 1). Protocol biopsies as early as 11 months and up to day 731 stained positive for C4d(16), but did not have features of active rejection. However, his five-year protocol biopsy revealed glomerular basement membrane duplication by light and electron microscopy with capillaritis and continued C4d deposition with minimal interstitial fibrosis (Fig.

3I). Although his renal function was stable with serum creatinine 1.6 at that time (Fig. 2), MMF was initiated 6 years after IS had been withdrawn. Since proteinuria became detectable after 7 years post-transplant and his kidney function has been slowly deteriorating, a brief course of IVIG and rituximab were administered and MMF has recently been switched to belatacept (Fig. 2) to prevent further progression of chronic rejection. Subject #5 remained stable for 5 years (Fig. 2) with no evidence of rejection or C4d deposition, despite intermittent detection of weak DSA after 3 years (Fig.4). Subsequently, after suffering multiple severe episodes of gout, his renal function deteriorated and C4d deposition transiently became detectable in the biopsy at 6 years. Despite a protocol biopsy at 6.3 years that showed minimal transplant glomerulopathy (cg1) (Figs. 3J, K) with negative C4d staining (Fig. 3L), renal function became unstable with serum Cr. level 2.3-2.8 mg/dl after the 7<sup>th</sup> year (Fig. 2) and Belatacept has recently been initiated.

**Clinical course of subjects in ITN036 (Subjects #6-10):** Subject # 6 developed the typically observed AKI after day 10 and recovered without additional treatment. His IS was slowly tapered and completely discontinued at 8 months after CKBMT. He is currently doing well with a serum Cr. level of 1.5 mg/dl for almost 5 years after transplantation, without ongoing IS (Fig. 2). His biopsies have shown no evidence of rejection (Figs.3M and N) but showed de novo C4d deposition in a biopsy taken at 2 years (Fig.3O). Immunosuppressive therapy for Subject #7 was discontinued at 8 months. She is currently 4.7 years

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after CKBMT with normal kidney function (serum Cr. 0.8 mg/dl) (Fig. 2) with no evidence of rejection or C4d staining in the 2-year protocol biopsy (Figs. 3P-R). Subject # 8 failed to recover from AKI. Biopsy performed on day 22 revealed arterial intimal matrix expansion with infiltrating mononuclear cells and arterial fibrinoid necrosis without C4d deposition in peritubular capillaries. No DSA was detectable. Differential diagnosis included acute cellular rejection type III versus intra-renal thrombotic microangiopathy, possibly due to tacrolimus toxicity. Tacrolimus was discontinued and three doses of anti-thymocyte globulin were administered with MMF. Despite these treatments, her kidney function gradually failed and she was returned to CAPD at 7 months after transplantation. Subject #9 developed a transient AKI after day 10 and recovered without additional treatment. Her immunosuppression was slowly tapered and was discontinued at 8 months after transplantation. She is currently well at almost 4.3 years after CKBMT with normal kidney function (serum Cr. 1.0 mg/dl)(Fig. 2). The protocol biopsy at 2 years showed no evidence of rejection (Figs.3S-U). In Subject #10, kidney function gradually returned to normal by 2 months after AKI and a 6-month protocol biopsy did not show rejection. His immunosuppression was discontinued at 8 months. One month later, he developed acute allograft pyelonephritis with moderately elevated serum Cr. (2.2 from 1.6 mg/dl). This was treated with antibiotics and the kidney function recovered to base line. However, 3 weeks after the resolution of his infection, he developed severe acute cellular rejection (Banff 2B)(Fig. 3V) with no C4d deposition (Fig. 3X). Several DSA assays were negative. He was treated with steroid pulses and anti-thymocyte

globulin, following which his renal function improved but never fully recovered (Fig. 2). A renal biopsy 6 months later showed interstitial and intimal fibrosis without active inflammation (Fig. 3W). His kidney function remained compromised thereafter and he recently received a successful second kidney transplant with conventional IS at 3 years after CKBMT.

**Anti-donor T cell responses:** MLR, CML, CTLp and HTLp assays in NKD03 subjects were previously reported.(16, 21) All NKD03 subjects developed donor specific nonresponsiveness (DSN) by 3 to 9 months in these assays. Serial in vitro immunological assays to test anti-donor T cell responses were also performed in the four ITN036 subjects who discontinued their immunosuppression. In these subjects, DSN or donor-specific hyporesponsiveness (DSH) also developed in MLR by 3 to 18 months, but anti-donor responses were again evident at 18-36 months in Subjects #7 and #9 (Table 2).

**High incidence of DSA production in the modified NKD03 subjects:** DSA has never been detected in Subjects #1, 6, 7, 8, 9 and 10. DSA was transiently detected only once in Subject #2 with no evidence of rejection. Anti-HLA class II DSA has been persistently positive in Subject #4, and was associated with persistent C4d deposition and glomerulopathy. In Subject #5, weak anti-class II DSA has been detected intermittently after 3 years with transient C4d deposition (Fig. 3). In ITN036 subjects, no DSA has been detectable even in Subject #10 who developed severe acute cellular rejection.

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**B cells (CD3-CD19+) were almost completely eliminated from the circulation for 6 months in ITN036 subjects:** Recovery of CD3<sup>-</sup>CD19<sup>+</sup> cells in the two subjects (#1 and #2) after treatment with the original NKD03 regimen was observed by days 50 and 100, respectively. With two doses of pre-transplant rituximab (modified NKD03), depletion of CD3<sup>-</sup>CD19<sup>+</sup> cells was extended to day 150 (Subjects #4 and #5) but complete loss of CD3<sup>-</sup>CD19<sup>+</sup> cells from the peripheral blood was not observed. In contrast, peripheral blood CD3<sup>-</sup>CD19<sup>+</sup> cells were less than 1-2/mm<sup>3</sup> for 6 months in all five ITN036 subjects treated with 4 doses of peritransplant rituximab (Fig. 45). (20)

**High serum BAFF levels in the recipients treated with rituximab:** Serum BAFF levels were less than 2 ng/ml at all time points assessed in Subject #1. Subject #2 had high BAFF levels initially until day 100 and humoral rejection was suspected at around day 45, when his BAFF level was highest (Fig. 5B, arrow). BAFF levels waned thereafter to less than 2 ng/ml after day 100 and he successfully discontinued his IS. In contrast, high BAFF levels were detected over 300 days in two subjects treated with the modified NKD03 regimen. Persistently high BAFF was also detectable for more than 300 days in the ITN036 subjects (Fig. 4).

## DISCUSSION

We report here that induction of long-term (greater than 11 years) stable renal allograft function without maintenance immunosuppression can be achieved after induction of transient lymphohematopoietic chimerism in approximately one-half of recipients of HLA-mismatched CKBMT. Consistent with the fact that chimerism in these recipients was transient, there has been no GVHD observed in our preclinical (10, 11) or clinical studies (16), which is an attractive attribute of this approach for tolerance induction. Apparently, the mechanism of tolerance induction after transient chimerism in nonhuman primates and humans differs from that observed in murine mixed chimerism models, where central deletional pathways have been demonstrated in conjunction with chimerism that persists indefinitely. (12, 22). (9, 23) Nevertheless, our NHP studies have consistently demonstrated the necessity for DBM engraftment, resulting in at least transient measurable donor chimerism, especially in lymphoid lineages, for successful induction of tolerance (10, 11, 24). Our nonhuman primate studies have shown that co-stimulatory blockade, with agents directed against CD40/CD154 (11) or B7/CD28 (manuscript in preparation) were important in achieving consistent tolerance. In these clinical trials, we have used an anti-CD2 monoclonal antibody (MEDI507) which provides both T-cell depletion and co-stimulatory blockade via the CD2/LFA-3 interaction in our clinical protocol (16). Our preclinical studies have also emphasized that the kidney allograft itself plays a critical role in the induction and maintenance of tolerance. In our studies in NHP, the same conditioning regimen that achieves

renal allograft tolerance after induction of transient chimerism, has consistently failed to induce tolerance of isolated heart allografts in monkeys(25). However, heart allograft tolerance is regularly achieved by co-transplanting the kidney from the same donor(26). Moreover, patients who lost chimerism after receiving the same regimen as NKDO3 and BMT for hematologic malignancies, but without a kidney transplant, failed to develop donor-specific hyporesponsiveness (19), in marked contrast to those receiving kidney transplantation with the same BMT regimen (21). We hypothesize that generation of donor-specific regulatory cells in the thymus during the original chimeric state ~~is a process that is maintained by combined with~~ continued interactions s of recipient T cells with as yet undefined donor cells or antigens in the kidney allograft ~~and~~ is responsible for maintenance of the tolerance (21). One potentially important renal cell is the renal tubular epithelial cell, that has been reported to participate in the induction of allospecific tolerance in rodents and, by *in vitro* assays in humans (27-29). Studies are in progress in our laboratories to assess the possible role of this cell in the NHP tolerance model upon which these clinical studies are based.

Despite the encouraging results of successful long-term IS-free renal allograft survival in our initial clinical trials, obstacles remain before we anticipate more widespread application of this approach. The first obstacle is acute kidney injury (AKI) that has been observed at approximately day 10 in all subjects, although minimally in Subject #1. We have previously reported this complication as “engraftment syndrome” which has been described previously following both autologous and allogeneic bone marrow transplantation (30). However,



“engraftment syndrome” may not adequately describe the phenomenon we have observed, since it was always associated both with recovery of host hematopoietic cells, potentially including homeostatic recovery of memory T cells, and with rapid loss of chimerism. We have noted that the AKI of engraftment syndrome has never been observed in our MHC-mismatched NHP model, in which chimerism is also lost, but at a more gradual pace, usually over 2-3 months(10, 11, 31). Our current interpretation of this observations is that the pre-transplant cyclophosphamide-based regimen chosen for the clinical trials(16), in place of low dose total body irradiation (TBI) used in the NHP preparative regimen(10, 11, 31, 32), may be less effective in preventing rapid homeostatic recovery of host memory T cells, which, when rapidly expanding, may have had destructive effector function at the level of donor renal endothelial cells. This hypothesis is consistent with the observation of CD8 T cell activation immediately following conditioning (18) and the appearance of CD8 T cells in peritubular capillary endothelial cells during the period of AKI (18). For this reason, in a current modification of our CKBMT protocol, we are testing the effect of substituting a low dose of TBI for pre-transplant cyclophosphamide (in progress).

Also, although the phenomenon of engraftment syndrome has been observed in autologous and allogeneic bone marrow transplantation [ ], the AKI observed in those cases was much milder than that observed in our protocol. One likely reason for this difference is that in the former cases, the recipients had normal kidneys, while the recipients of our CKBMT protocol had also just

received a kidney transplant, with attendant inflammatory changes which would likely make it more susceptible to any additional injury during the early post-transplant period. Concurrent calcineurin inhibitor toxicity would undoubtedly also contribute to renal dysfunction, and it is likely that the combination of tacrolimus toxicity and engraftment syndrome contributed heavily to the renal failure from TMA observed in Subject # 8. In our modified protocol, we will therefore discontinue treatment with calcineurin inhibitors during the duration of engraftment syndrome, should it occur despite the change from cyclophosphamide to irradiation in the preparative regimen.

Another obstacle, observed especially in the modified NKD03 subjects, was the frequent development of either transient or persistent DSA, despite specific loss of anti-donor T cell responses. As depletion of B cells enhances B cell activating factor (BAFF) production which has been found to significantly enhance the differentiation of marginal zone B cells into immunoglobulin secreting cells in a T cell dependent manner(33), we measured peripheral blood B cells (CD19+) and serum BAFF levels after transplantation. In Subject #2, humoral rejection, suspected around day 45 while tapering IS, was associated with high BAFF levels (>8 ng/ml). His BAFF levels subsequently waned to the baseline level by day 100 and IS was successfully withdrawn without DSA development. In the two modified NKD03 subjects, B cells were not completely depleted and recovered within 6 months. In addition, persistently high serum BAFF levels were detected in these patients, which may have contributed to

enhanced DSA production. In contrast to the results in the modified NKD03 protocol, the intensified anti-B cell treatment included in the ITN036 regimen was associated with almost no evidence of DSA production. In these ITN036 subjects, although BAFF levels were elevated similarly to those observed in the modified NKD03 patients, peripheral blood B cells were essentially nondetectable for more than 6 months and there was no development of DSA. Thus, high BAFF levels may not be a risk factor for DSA production if B cells are profoundly depleted.

Although anti-HLA antibodies are thought to represent T cell-dependent responses, we found no apparent relationship between DSA production and anti-donor T cell responses in these clinical studies. Thus, modified NKD03 subjects developed DSA despite undetectable anti-donor T cell responses and Subjects #7 and #9 in ITN036 have not developed DSA despite persistent anti-donor CTL responses. Our clinical observations therefore suggest that B cell depletion for 6 months by rituximab, as achieved in the ITN036 subjects, may avoid the DSA obstacle to tolerance induction via the mixed chimerism approach. In this regard, we are currently investigating a conditioning regimen that combines B cell depletion and anti-BAFF antibody in nonhuman primates.

In conclusion, our clinical studies have shown that immunosuppression-free stable renal allograft function can be achieved in HLA-mismatched donor-recipient pairs, with follow up times of greater than 11 years, by induction of transient chimerism through DBMT. We hope that the relatively small modifications of the protocol we have described will avoid the remaining

obstacles to more widespread application of this technology for clinical tolerance induction.

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**Disclosure:**

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation. This manuscript was also not prepared or funded by any commercial organization.

## Figure Legends

### **Fig. 1:** Nonmyeloablative conditioning regimens.

The initial conditioning regimen (Fig. 1, NKD03) consisted of cyclophosphamide (60 mg/kg/d) administered i.v. on days –5 and –4 with respect to transplantation; humanized anti-CD2 mAb (MEDI 507, MedImmune Inc., Gaithersburg, MD) (0.6 mg/kg/dose) on days -2, -1, 0 and +1; cyclosporine A (CyA, Novartis Pharmaceuticals Inc., East Hanover, NJ) (5 mg/kg) i.v. on day –1; and thymic irradiation (700 cGy) on day –1. Hemodialysis was performed before and 14 hours after each dose of cyclophosphamide. On Day 0, kidney transplantation was followed by i.v. infusion of unprocessed donor bone marrow (DBM). Oral CyA (Neoral, Novartis Pharmaceuticals, Inc.) was administered postoperatively at 8 to 12 mg/kg/day with target trough blood levels of 250-350 ng/ml, then tapered and discontinued over several months.

The protocol was modified after treatment of the third subject (see Results), with the addition of Rituximab, 375 mg/m<sup>2</sup>/dose on days -7 and -2; and prednisone, 2 mg/kg/d starting on the day of transplantation and tapering to withdrawal over the next 10 post-transplant days (modified NKD03). Since subjects treated with this modified NKD03 still developed donor specific antibodies (DSA) after discontinuation of immunosuppression, the regimen was further modified (Fig.1) to add two more doses of Rituximab (375 mg/m<sup>2</sup>/dose) on days 5 and 12, plus a more prolonged course of prednisone until day 20, and tacrolimus in place of CyA (ITN036). Tacrolimus was slowly tapered over several months and completely discontinued at 8 months after confirming no rejection by a 6 month

protocol biopsy. All treatment regimens were approved by the Massachusetts General Hospital Institutional Review Board (IRB) and developed in collaboration with the Immune Tolerance Network (ITN).

The initial preparative conditioning regimen (NKD03) consisted of cyclophosphamide (60 mg/kg/d) on days -5 and -4, humanized anti-CD2 mAb (0.6 mg/kg/dose) on days -2, -1, 0 and +1; cyclosporine A targeting trough levels of 250-300 mg/ml beginning on day -1; and thymic irradiation (7Gy) on day -1. Kidney transplantation was followed by i.v. infusion of donor bone marrow (DBM). CyA was tapered and discontinued over 9-14 months.

In the modified NKD03 regimen, administration of Rituximab (375 mg/m<sup>2</sup>/dose) on days -7 and -2 and a short course of post-transplant prednisone were added.

In the ITN036 regimen, two more dose of Rituximab (375 mg/m<sup>2</sup>/dose) on days +5 and +12 and a longer course of prednisone were added and post-transplant cyclosporine was replaced with an 8 months course of tacrolimus, targeting trough levels of 8-12 ng/ml.

**Fig. 2:** Clinical course after CKBMT

Blue lines indicate serum Creatinine levels (mg/dl) after CKBMT. Green bars indicate induction immunosuppression (CyA:cyclosporine, Tac:tacrolimus).

Orange /blue bars indicate reinstitution of maintenance immunosuppression (MMF: mycophenolate mofetil, actino: actinomycin, Pred: prednisone), CTLA4Ig (belatacept).

**Fig. 3:** Late renal allograft biopsies (all protocol biopsies unless noted).

**A-C (Subject #1 at 7.5 years):** Protocol biopsy shows no evidence of rejection by light microscopy (LM) (A). The glomeruli are normal by electron microscopy (EM) (B) and no C4d staining is detectable by immunofluorescence (IF) (C)

**D, E, F (Subject #2 at 8 years):** An indication biopsy for proteinuria shows prominent lobular mesangial expansion with widespread glomerular basement membrane (GBM) duplication (D, arrow). Granular dense deposits in the duplicated GBM are seen by EM (E). Granular and segmental staining for C3 along the GBM and in the mesangium is evident by IF, but no immunoglobulin was detected (F), indicative of recurrent C3 glomerulopathy (originally classified as MPGN, type I)..

**G,H,I (Subject #4 at 5 years):** Widespread GBM duplication (arrow) and glomerulitis is seen by LM (G). EM shows prominent duplication of the GBM without deposits and reactive endothelial cells (H). C4d deposition is detected in peritubular capillaries (I). These findings are indicative of chronic, active antibody mediated rejection, which at this time was subclinical.

**J,K,L (Subject #5 at 6 years):** The biopsy shows minimal glomerulitis by LM (j), slight segmental GBM duplication by LM (J) and EM (K) and no C4d deposition by IF (L)

**M,N,O (Subject #6 at 2 years):** LM shows normal glomeruli with rare foci of interstitial mononuclear inflammation affecting <5% of the cortex (M). EM shows minimal focal GBM duplication and normal endothelium (N). C4d was present in the majority of the peritubular capillaries (O).



**P,Q, R (Subject #7 at 2 years):** LM is within normal limits (P). EM reveals a normal GBM and endothelium; foot process effacement is present in a minority of the capillaries (20%) (Q). There is no C4d deposition (R).

**S,T, U (Subject #9 at 2 years):** The kidney biopsy is within normal limits and shows no evidence of rejection by LM (S) with normal glomeruli by EM (T). There is no evidence of recurrent MPGN. No C4d is detected (U).

**V,W,X: (Subject #10):** Indication biopsy at 9.5 months shows acute cellular rejection with endarteritis (V). A protocol biopsy six months later shows complete resolution of the inflammatory process and residual interstitial and intimal fibrosis (W). No C4d deposition is detectable at 9.5 months (X) or at other times.

Light microscopy stains: H&E, A, M, V; PAS, D, G, J, P, S. Immunofluorescence stains: C4d, C, F, I, L, O, R, U, X.

#### **Fig. 4: Detection of donor specific antibody (DSA)**

DSAs are detected by ELISA.

NKD03: Subject #1: DSA never detected. Subject #2: DSA transiently detected once at 7 years. Subject 3: Although pre-transplant DSA was negative by ELISA, retrospective Luminex showed positive anti-class I DSA (B44) before transplantation. The subject developed acute humoral rejection on day10 with DSA (B44 and DR4).

Mod NKD03: Subjects 4: Anti-donor DR17 antibody has been persistently positive since soon after stopping immunosuppression. Subject 5: Weak antidonor DR53 antibody has been intermittently detectable.

ITN036: In contrast to NKD03 subjects, No DSA has been detectable in any ITN036 subject including Subject #10 who developed severe acute cellular rejection.

**Fig. 5:**

**A: CD3<sup>+</sup>CD19<sup>+</sup> cell depletion after CKBMT**

In two NKD03 subjects, recovery of CD3<sup>+</sup>CD19<sup>+</sup> cells (B cells) was found by day 50 (Subject #1) and day 120 (Subject #2)(gray lines). With addition of two doses of pre-transplant rituximab (modified NKD03), B cells were partially depleted and recovered by days 150 and 180 (blue lines). With 4 doses of peri-transplant rituximab (ITN036), B cells were nearly completely depleted (less than 1-2/mm<sup>2</sup>) for 180 days..

**B: Serum BAFF**

Serial serum BAFF levels were measured by ELISA. Serum BAFF levels were less than 2 ng/ml at all time points assessed in Subject #1. Subject #2 had high BAFF levels initially until day 100 and humoral rejection was suspected at around day 45, when his BAFF level was highest (Fig. 5B). BAFF levels waned thereafter to less than 2 ng/ml after day 100 and he successfully discontinued his IS. In contrast, high BAFF levels were detected over 300 days in two subjects treated with the modified NKD03 regimen. Persistently high BAFF was also detectable for more than 300 days in the ITN036 subjects.

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